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Characterization of male germ cell markers in canine testis

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ABSTRACT

Spermatogenesis begins at puberty and continues throughout a male's life. This process is initiated and maintained by spermatogonial stem cells in the seminiferous tubules, and these cells produce haploid spermatozoa. Markers of male germ cells have been fully identified in rodents, including mice and rats, but not in canines. To characterize the canine male germ cells, histological and immunohistochemical analyses were performed, using prepubertal (1–3-month-old), early pubertal (4-month-old), and postpubertal (7-month-old) dog testes. Expression of protein gene product 9.5 (PGP9.5), deleted in azoospermia-like (DAZL), synaptonemal complex protein (SCP3), tyrosine-protein kinase Kit (C-kit), and acrosin was confirmed by immunohistochemical analysis. PGP9.5 and DAZL were detected in spermatogonia and co-localized near the basement membrane of seminiferous tubules. Some SCP3-positive cells expressed PGP9.5 but not C-kit, and most of these cells were located near the basement membrane. C-kit is a marker of differentiated spermatogenic cells. In addition, acrosin was detected in C-kit-positive spematocytes and mature spermatozoa, whereas C-kit was detected in Sertoli cells in all stages of canine testis development. We suggest that male germ cell markers detected in other species are conserved in canines. PGP9.5, DAZL, SCP3, and acrosin expressions were conserved among various species, but C-kit expression varied. This study might facilitate the identification of stage-specific canine germ cell markers and cellular mechanisms of spermatogenesis.

1. Introduction

Spermatogonial stem cells (SSCs) are stem cells in the testis, which generate haploid mature sperm during a male's life, after puberty (Aponte et al., 2005). In rodents, specific biomarkers of male germ cells have been identified, but in canines they are not yet fully discerned. Previous studies have shown that canine SSCs can be identified using protein gene product 9.5 (PGP9.5), Vasa, deleted in azoospermia-like (DAZL), promyelocytic leukemia zinc finger protein, and AP180 as markers (Harkey et al., 2013; Lee et al., 2014). However, stage-specific markers of canine male germ cells have not been studied until date.

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Ubiquitin-dependent proteolysis has been implicated in the control of mammalian gametogenesis, and PGP9.5 (or ubiquitin carboxy-terminal hydrolysis L1) is a deubiquitinating enzyme (Sutovsky, 2003). In testes, PGP9.5 has been demonstrated to be expressed in spermatogonia of various species, including mice (Kwon et al., 2004), bulls (Wrobel, 2000), goats (Heidari et al., 2012), dogs (Harkey et al., 2013; Lee et al., 2014), and pigs (Lee et al., 2013; Luo et al., 2006).

DAZL is a germ cell-specific, RNA-binding protein that is considered a major regulator of spermatogenesis (Collier et al., 2005). Expression of DAZL has been reported in male germ cells of several species, including mice (Ruggiu et al., 1997), pigs (Luo et al., 2010), and bulls (Zhang et al., 2008). During spermatogenesis, DAZL is expressed in the nucleus of spermatogonia and transfers to the cytoplasm of primary spermatocyte during meiosis (Reijo et al., 2000).

Synaptonemal complex protein 3 (SCP3) is a meiosis-specific structural protein that appears at axial and lateral elements of the synaptonemal complex (Parra et al., 2004). Localization of SCP3 has been reported in surface-spread rodent spermatocytes (Lammers et al., 1994). It first appears at axial elements in leptotene spermatocytes and is found at lateral elements during pairing and synapsis during zygotene and pachytene stages, respectively. In diplotene spermatocytes, SCP3 is present at desynapsed lateral elements and persists between sister chromatids until the metaphase I/anaphase I transition (Dobson et al., 1994; Moens and Spyropoulos, 1995).

C-kit is a marker of SSC potency loss and its expression continues until meiosis is initiated (Schrans-Stassen et al., 1999). *c-kit* is allelic to the W locus on mouse chromosome 5 (Chabot et al., 1988). Its expression is detectable in preleptotene spermatocytes and type A, intermediate, and type B spermatogonia, but not in undifferentiated spermatogonia (Yoshinaga et al., 1991). However, whether C-kit is present in both undifferentiated and differentiating type A spermatogonia or only in the latter, is still under debate.

Sperm acrosin is a trypsin-like serine proteinase present in acrosomes. It functions in sperm binding and penetration of oocyte zona pellucida. Acrosin expression during spermatogenesis was studied using mouse testes as a model. Immunocytochemical analysis revealed that step 9 spermatids were the first cells to react with C11H antibody (Kallajoki et al., 1986). During late step 15, the antigen was located at a site in the acrosome, which is typical of step 16 spermatids and spermatozoa (Kallajoki et al., 1986).

In this study, we described the stage-specific canine male germ cell markers. To substantiate our study, we identified PGP9.5, DAZL, SCP3, C-kit, and acrosin expressions in canine testes during development and compared the expression of these germ cell markers with those in adult canine testes by co-immunostaining.

2. Materials and methods

2.1. Experimental design

Beagle testes were obtained from routine castrations performed by veterinary surgeon. Tissues were immediately stored on ice and processed within 12 h of surgery. For each stage (prepubertal, 1–3-month-; pubertal, 4-month-; and postpubertal, 7-month-old; n = 3 each), a portion of a testis was fixed in Bouin's solution (HT10132; Sigma-Aldrich; St. Louis, MO, USA) overnight, at 4 °C. Another portion was stored at −80 °C for western blot analysis. Experimental procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Konkuk University (approved No. KU16066).

2.2. Histology

Samples were subsequently washed with 70–100% (v/v) ethanol, embedded in paraffin, sliced into 5-μm thick sections, using a microtome (Thermo; Barrington, IL, USA), and mounted on glass slides. The mounted canine testis tissue was rehydrated with xylene and 100–0% ethanol. The rehydrated testes were stained for nucleus and cytoplasm, with hematoxylin and eosin (H&E).

2.3. Immunohistochemistry

To identify the localization of PGP9.5-, DAZL-, SCP3-, C-kit-, and acrosin-expressing cells in the canine testis, the antigen was retrieved by boiling the tissue for 30 min in Tris-ethylenediaminetetraacetic acid (Tris-EDTA) solution (10 mM Tris base, 1 mM EDTA, and 0.05% Tween-20; pH 9). The membranes of the antigen-retrieved tissues were permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100, for 10 min. Non-specific protein binding was blocked with 2% bovine serum albumin in PBS for 1 h at room temperature (RT). Tissues were incubated overnight at 4 °C with the following primary antibodies: PGP9.5 (1:1000 dilution; 7863-1004; AbD Serotec; Raleigh, NC, USA), DAZL (1:50 dilution; SC-27333; Santa Cruz Biotechnology, Inc.; Dallas, Texas, USA), SCP3 (1:50 dilution; SC-33195; Santa Cruz Biotechnology, Inc.), C-kit (1:50 dilution; SC-1494; Santa Cruz Biotechnology, Inc.), and acrosin (1:50 dilution; SC-51504; Santa Cruz Biotechnology, Inc.). After washing three times with PBS, the appropriate secondary antibody was added and incubated for 2 h at RT. Peroxidase substrate detection kit (SK-4100; Vector Laboratories; Burlingame, CA, USA) was used for the detection of putative canine germ cell markers, according to the manufacturer's instructions. To identify nuclei, 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich; D9542) was added at a concentration of 1 μg/ml for 10 min. Finally, mounting solution (Dako, Carpinteria, CA, USA; S3025) was used to fix the immunostained canine testis tissues. The co-immunostained tissues were observed under a confocal microscope (Carl Zeiss, Oberkochen, Germany; LSM 700).

2.4. Western blot analysis

Total protein from canine testes was isolated using a Proprep kit (iNtRON, Seongnam, South Korea), according to the manufacturer's instructions. Fifty micrograms of total protein from each sample was separated by 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk and incu-